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Mixtures of aromatic compounds induce ligninolytic gene expression in the wood-rotting fungus *Dichomitus squalens*

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Abstract

Heterologous production of fungal ligninolytic cocktails is challenging due to the low yields of catalytically active lignin modifying peroxidases. Production using a natural system, such as a wood-rotting fungus, is a promising alternative if specific or preferential induction of the ligninolytic activities could be achieved. Using transcriptomics, gene expression of the white-rot *Dichomitus squalens* during growth on mixtures of aromatic compounds, with ring structures representing the two major lignin sub-units, was compared to a wood substrate. Most of the genes encoding lignin modifying enzymes (laccases and peroxidases) categorised as highly or moderately expressed on wood were expressed similarly on aromatic compounds. Higher expression levels of a subset of manganese and versatile peroxidases was observed on di- compared to mono-methoxylated aromatics. The expression of polysaccharide degrading enzymes was lower on aromatic compounds compared to wood, demonstrating that the induction of lignin modifying enzymes became more specific. This study suggests potential for aromatic waste streams, *e.g.* from lignocellulose pretreatment, to produce a lignin-specific enzyme cocktail from *D. squalens* or other white-rot fungi.

Keywords

Basidiomycete, aromatics, lignin, gene expression, white-rot

1. Introduction

When growing on lignocellulose, white-rot fungi (WRF) express a diverse array of genes encoding lignin and polysaccharide degrading activities (Mäkelä et al., 2014; Peng et al., 2018; Rytioja et al., 2014). WRF use class II heme peroxidases, accessory enzymes for H_2O_2 production and probably laccases to degrade lignin which structurally consists of two major sub-units, guaiacyl (G) and syringyl (S). During this process, derivatives of G-related, mono-methoxylated compounds, such as vanillic acid, and S-related di-methoxylated compounds, such as syringic acid, can be released (Chen et al., 1982; Daly et al., 2018; Henderson, 1955). Ligninolytic enzyme cocktails have potential for enzymatic pretreatment of lignocellulose (Schroyen et al., 2015) as well as generating lignin-derived precursors of aromatic building blocks (Abdelaziz et

al., 2016; Lubbers et al., 2019). Heterologous production of fungal ligninolytic cocktails is challenging because of the low yields of catalytically active lignin modifying peroxidases (Lambertz et al., 2016). Therefore, induction of ligninolytic enzymes in a natural system, such as a wood rotting fungus, is an attractive alternative.

As well as aromatics (Manubens et al., 2003; Moiseenko et al., 2018), other factors including metal ions, can affect ligninolytic gene expression and enzyme production. E.g. copper has been shown to induce expression of laccases and manganese peroxidases in *Phanerochaete chrysosporium* (Alvarez et al., 2009), whereas manganese is required for production of manganese peroxidases in *Dichomitus squalens* (Perie et al., 1996). Excess carbon and nitrogen can also repress the expression of ligninolytic genes (Janusz et al., 2013). In contrast to ligninolytic genes, sugar molecules are the main inducers of polysaccharide degrading enzymes; *e.g.* cellobiose is a major inducer in *D. squalens* (Casado López et al., 2018).

A better understanding of the role of G- and/or S-related aromatics in the induction of ligninolytic genes in white-rot fungi is crucial to move towards industrial production of the corresponding enzymes. It would provide the necessary insights as to whether lignocellulose-derived waste streams (Kim, 2018) that contain varying amounts of G- and/or S-related aromatics could be used as substrates for white-rot fungi to produce ligninolytic enzymes.

The white-rot basidiomycete *D. squalens* colonizes both softwood and hardwood in nature (Krah et al., 2018) and contains a full spectrum of genes encoding ligninolytic enzymes in its genome (Casado López et al., 2019). In this study, we investigated if aromatic compounds could induce ligninolytic gene expression in *D. squalens* and if the number of methoxylated groups on the aromatic ring affected expression levels of ligninolytic genes.

2. Materials and methods

The materials and methods section is available in the online supporting information.

3. Results and discussion

3.1 *D. squalens* showed distinct transcriptome patterns on aromatic mixtures compared to wood

There were distinct global transcriptome patterns from *D. squalens* mycelium grown for five days on birch wood compared to G- and S-lignin related mixtures of aromatic compounds, as shown by the three separate clusters in the principal component analysis (Figure S1). The G-lignin related mixture contained guaiacol, vanillin, vanillic acid and ferulic acid, and the S-lignin related mixture contained syringol, syringic acid, sinapic acid and syringaldehyde, with each aromatic at a 50µM concentration. Similarly, when the plant biomass degrading CAZymes were analysed by hierarchical clustering, the samples formed three distinct clusters based on the used substrates (Figure S2). The radial growth of *D. squalens* was greater on the G-related compared to the S-related aromatic mixture whereby the colony diameter was ~10% smaller on the latter (Figure 1 and Figure S3).

3.2 Expression of ligninolytic activity encoding transcripts on aromatics and wood

The *D. squalens* wood culture was used to compare the expression of the ligninolytic transcripts on a natural substrate to the aromatic mixtures. On the wood cultures, a quarter of the lignin modifying enzymes (LMEs) and half of the H_2O_2 supplying enzymes encoding genes were highly or moderately expressed. Most of these were also highly or moderately expressed on the aromatic mixtures, illustrated by clusters with similar levels on all conditions (*e.g.* cluster 12) or higher on aromatic mixtures (clusters 10 and 11) (Figure S2). The expressed gene set on the S-related aromatics was a better match to that of wood, *e.g.* by high expression of a versatile peroxidase *vp3* that was lowly expressed on G-related aromatics. *Vp3* was also lowly expressed when *D. squalens* was exposed separately to vanillin, vanillic acid and ferulic acid (Kowalczyk et al., 2019), which represent three out of the four aromatic compounds from the G-related mixture. Versatile peroxidases are important as they can directly oxidise the lignin polymer (Sáez-Jiménez et al., 2016), unlike the manganese peroxidases (Hofrichter, 2002). From the total expression of ligninolytic transcripts, there was a clear effect of S-related aromatics on total expression of LMEs, but little difference on H_2O_2 supply (Figure 2A).

3.3 Differentially expressed transcripts on G- compared to S-related aromatics

LMEs and H_2O_2 supply related transcripts were higher on the S- than G-related aromatics, including transcripts of *D. squalens mnp2*, *mnp7*, *mnp9*, *vp3* and *lcc3* (Figure 2B and Table S1H). These transcripts are good candidates to investigate whether their encoded LMEs have preferential activities towards S-related aromatics. Previously, syringic acid increased the expression of an *mnp* in the white-rot fungus *Ceriporiopsis subvermispora* (Manubens et al., 2003) and of multiple laccases in the white-rot fungus *Trametes hirsuta* (Moiseenko et al., 2018). The lack of LMEs or H_2O_2 supply related transcripts with higher expression levels on G-related compared to S-related aromatics may be explained by the presence of G-related ring structures in both cultures, *e.g.* by demethylation of the 5-position on the S-related aromatics. Differentially expressed genes encoding intracellular enzymes likely involved in detoxification processes included three cytochrome P450s (cytP450s) and two glutathione S-transferases (GSTs), and four cytP450s that were higher expressed on the S- and G-related aromatics, respectively (Figure 3). One of the cytP450 encoding genes that was higher on the G-related aromatics (*Dicsqu464_1_PID_950066*) was annotated as a CYP5150 member and the top BLASTp hit in *P. chrysosporium* (*Phchr2_PID_3023166*) at JGI's Mycocosm was shown to hydroxylate 4-propylbenzoic acid (Ichinose and Wariishi, 2012) and in another study had activity towards a broad range of structurally varied compounds where the protein was referred to as 121a (Hirosue et al., 2011). A cytP450 encoding gene annotated as a CYP5035 member (*Dicsqu464_1_PID_918682*) and another annotated as a CYP530 member (*Dicsqu464_1_PID_975696*) were higher expressed on the S-related aromatics. CYP5035 proteins from *P. chrysosporium* were found to oxidise a broad range of compounds (Hirosue et al., 2011; Syed et al., 2014) and CYP530 members are described as involved in degradation of fatty acids and hydrocarbons (Moktali et al., 2012). Four cytP450s, one higher on the S-related and three on the G-related aromatics, were all annotated with the CYP5144 family which is one of the largest families in basidiomycetes with activity towards a broad range of compounds (Syed et al., 2014). These genes are candidates to overexpress in *D. squalens* to potentially improve its tolerance to the aromatics and alleviate stress-related effects of aromatic compounds.

Several GO terms related to proteolysis, possibly related to the low nitrogen levels (Snyman et al., 2019), used to avoid repression of ligninolytic activities, were

enriched in transcripts that were higher on the S-related aromatics (Table S2). From the transcripts higher on the G-related aromatics, GO terms related to polysaccharide degradation were enriched although most of these polysaccharide degrading CAZymes were not highly expressed (Table S1E).

3.4 A higher specificity for induction of ligninolytic versus polysaccharide degrading activities was observed on aromatic compared to wood cultures

The total expression of polysaccharide-degrading CAZy genes was approximately three-fold significantly lower ($P<0.05$) on the aromatic mixtures compared to birch wood (Figure 4A). A small subset of polysaccharide-degrading CAZy genes was significantly higher expressed on the aromatic mixtures compared to the wood (Figure 4B), but their expression increased on average only ~2.5-fold. Aromatic compounds may make a minor contribution to the overall expression of the polysaccharide degrading CAZymes, as aromatic compounds have been shown to induce feruloyl esterases, which cleave linkages between xylan and lignin, in the ascomycete fungus *Aspergillus niger* (de Vries et al., 2002). However, the *D. squalens* feruloyl esterases were not amongst the transcripts higher expressed on the ferulic acid containing aromatic mixture nor was there induction of feruloyl esterases when *D. squalens* was cultured with ferulic acid as the sole aromatic compound (Kowalczyk et al., 2019).

In conclusion, aromatic compounds can induce expression of ligninolytic transcripts similar to wood substrates. This provides a basis for future investigation of *D. squalens* enzyme production using aromatic waste streams.

Declaration of interests

The authors declare no conflict of interest.

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Figure legends

Figure 1. Representative images of *D. squalens* cultures after 5 d of growth on (A) birch wood or (B) guaiacyl lignin-related or (C) syringyl lignin-related aromatic mixtures. The images of the inverted plates containing the aromatic compounds show the stronger colouration in the plate containing the syringyl lignin-related aromatics. Note that there was no noticeable colour change in the plates containing the birch wood cultures.

Figure 2. Lignin-related gene expression. (A) Total expression for lignin modifying enzymes and H_2O_2 supplying enzymes when *D. squalens* was grown on birch wood (BiW), guaiacyl (G) lignin-related or syringyl (S) lignin-related aromatics. (B) Expression level of genes encoding laccases or peroxidases that were moderately or highly expressed in at least one of the BiW, S and G conditions. LCC = laccase, MnP = manganese peroxidase and VP = versatile peroxidase. Error bars represent standard errors (n = 3).

Figure 3. Expression levels of genes encoding cytochrome P450s (CYP) or glutathione S-transferases (GST) differentially expressed when *D. squalens* was grown on guaiacyl (G) lignin-related or syringyl (S) lignin-related aromatics. The protein ID (PID) for each gene is shown along with either a cytochrome P450 family or GST annotation. Error bars represent standard errors (n = 3). The CYP family annotations are those assigned by the fungal cytochrome P450 database pipeline (Park et al., 2008).

Figure 4. Polysaccharide-degrading CAZy gene expression. (A) Total polysaccharide-degrading CAZy gene expression and total expression of genes acting on particular polysaccharide(s), and (B) number of significantly higher polysaccharide-degrading CAZy genes between the birch wood (BiW) or mixtures of guaiacyl (G) lignin-related or syringyl (S) lignin-related aromatics cultures. Error bars represent standard errors (n = 3).

Appendix A. Supplementary data

Figure S1. Principal component analysis (PCA) of RPKM values of all genes where there was expression > 0 in at least one biological replicate from *D. squalens* cultures on birch wood (BiW) or mixtures of guaiacyl lignin-related (G_aro) or syringyl lignin-related (S_aro) aromatics. Dim = dimension.

Figure S2. Hierarchical clustering, using Euclidian distance of transcript levels for genes encoding polysaccharide degrading CAZymes, lignin modifying enzymes and H_2O_2 supplying enzymes from each of the replicate *D. squalens* cultures containing birch wood (BiW), syringyl lignin-related aromatics (S_aro) or guaiacyl lignin-related aromatics (G_aro). The genes are colour-coded according to the substrate they act on or function in the case of H_2O_2 supply. Listed alongside the functional information for the genes is whether the mean transcript levels in each of the conditions was classified as low (L), moderate (M) or high (H). See Table S1 for explanation of the abbreviations used for the activities.

Figure S3. Colony diameter of *D. squalens* cultures growing on birch wood or mixtures of mono- (guaiacyl lignin-related) or di-methoxylated (syringyl lignin-related) aromatics. Error bars represent standard errors (n = 4).

Table S1. RNAseq dataset for *D. squalens* cultured on either birch wood (BiW), guaiacyl lignin-related aromatics (G_aro) or syringyl lignin-related aromatics (S_aro).

Table S2. List of gene ontology (GO) terms enriched in transcripts that were significantly higher in (A) the guaiacyl-lignin related aromatics culture and (B) the syringyl-lignin related aromatics culture when these *D. squalens* cultures were compared to each other.

Table S3. Sugar composition of the birch wood as measured from the acid hydrolysate and lignin composition from gel-state whole cell wall 2D-HSQC NMR analysis. Data was adapted from Daly et al. (2018).

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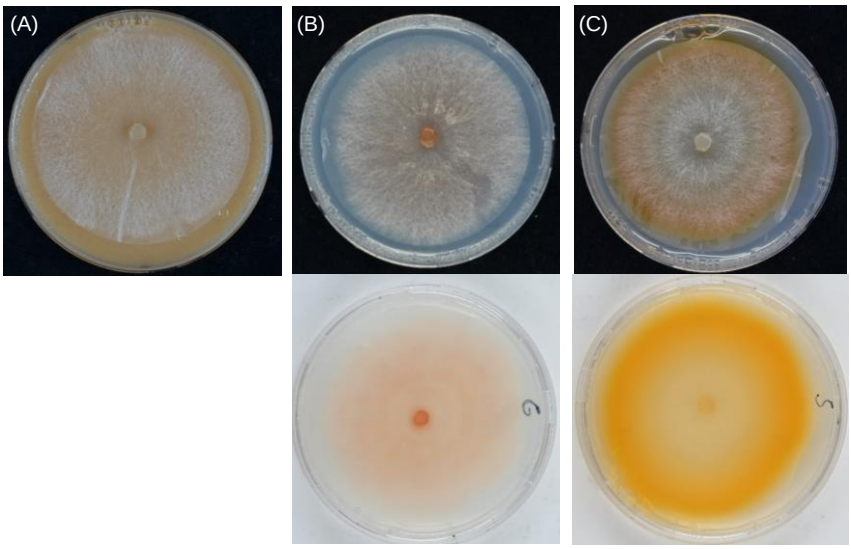

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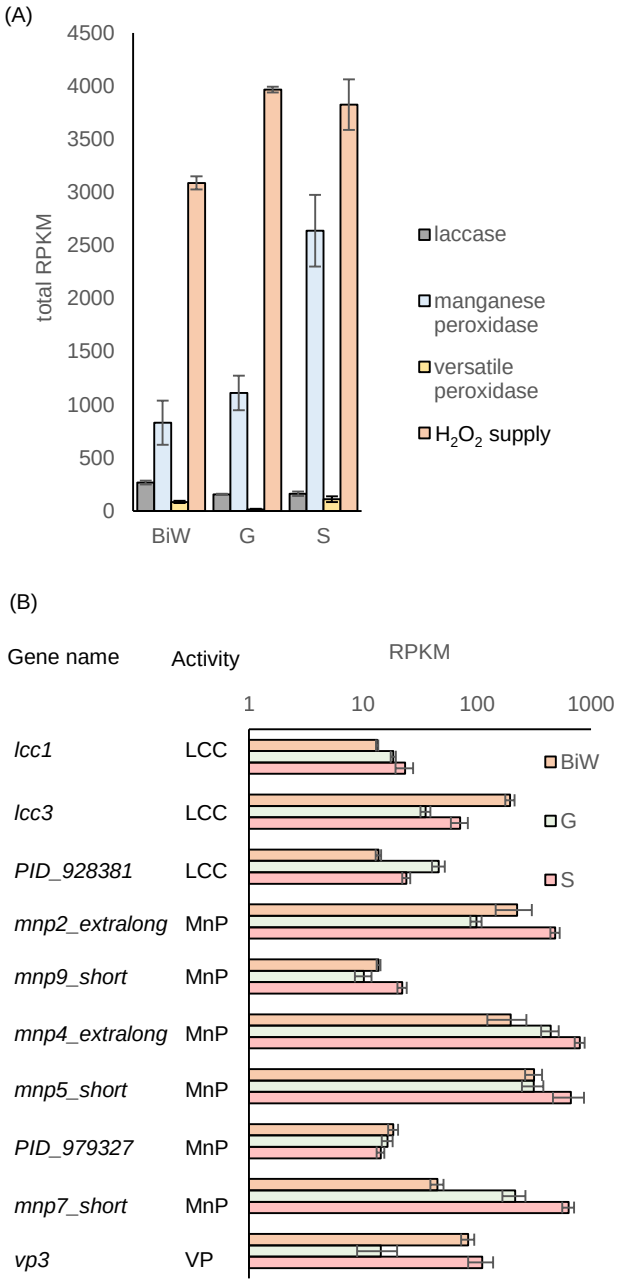

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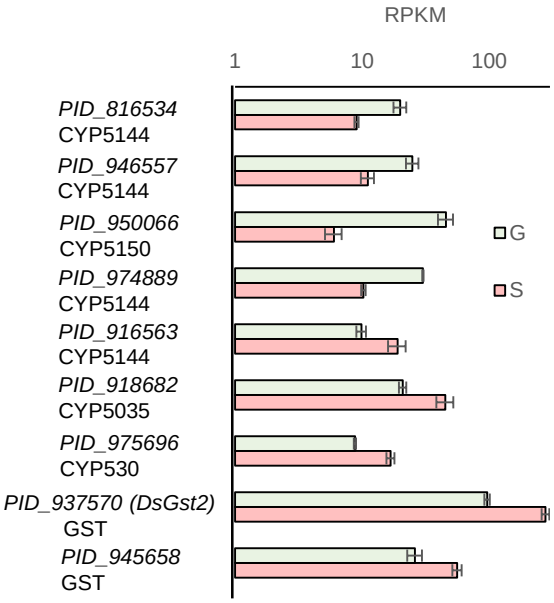

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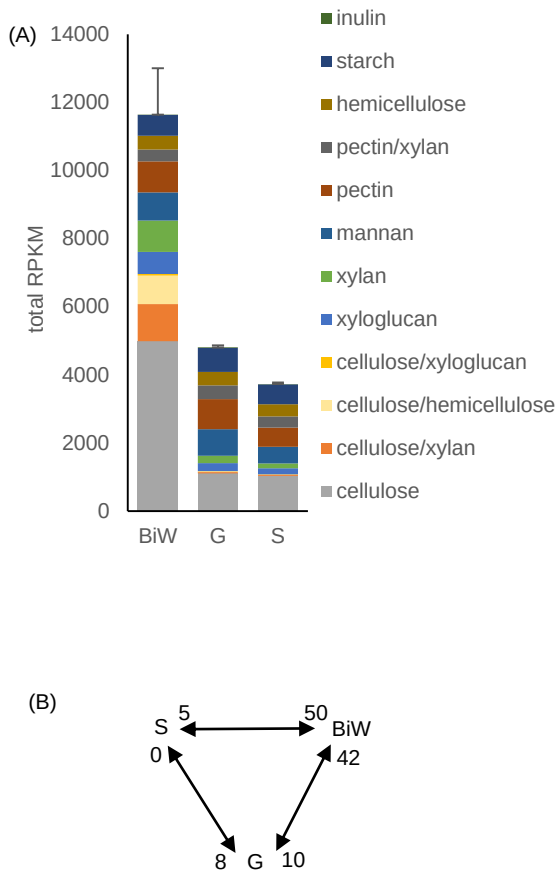

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